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- NISHIOKA, Toshimi
Hoechst Industry Limited
Tokyo 107 (JP)
- HOGA, Takuya
Hoechst Industry Limited
Tokyo 107 (JP)
- KUROKAWA, Nobuyuki
Tokyo 157 (JP)
- FUKUZAWA, Junichi
Hoechst Industry Limited
Tokyo 107 (JP)
- LAND, Horst-Tore
D-65719 Hofheim (DE)
- HELMER-METZMANN, Fredy
D-55270 Essenheim (DE)

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29.12.1995 JP 354063/95(71) Applicant:
Hoechst Research & Technology Deutschland
GmbH & Co. KG
65926 Frankfurt am Main (DE)(72) Inventors:

- NAKAMURA, Toru
Hoechst Industry Limited
Tokyo 107 (JP)

(54) HOT-ROLLER FIXING TONER FOR DEVELOPING ELECTROSTATICALLY CHARGED IMAGES

(57) This invention relates to a toner for developing an electrostatically charged image of a heat roller type copier or printer, the toner consisting essentially of a binder resin, a colorant and a charge control agent, in which the binder resin at least includes a polyolefin resin having a cyclic structure, and a polyolefin resin of a cyclic structure having an intrinsic viscosity (i.v.) of 0.25 dl/g or more, a heat distortion temperature (HDT) by DIN53461-B of 70 °C or higher, and a number average molecular weight of 7,500 or more and a weight average molecular weight of 15,000 or more, as measured by GPC, is contained in a proportion of less than 50% by weight based on the entire binder resin. The toner for developing an electrostatically charged image according to the invention is excellent in fixability, light transmission, and anti-toner spent properties, giving a sharp, high quality image, can be applied to any of a dry one-component magnetic toner, a dry one-component nonmagnetic toner, a dry two-component toner and a liquid toner, and exhibits marked effects particularly when used in a color toner.

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Description**Technical Field**

5 The present invention relates to a toner for developing an electrostatically charged image of a heat roller type copier or printer. More specifically, this invention relates to a dry one-component magnetic toner, a dry one-component non-magnetic toner, a dry two-component toner or a liquid toner which, when fixed, is excellent in anti-spent toner effect, and can form a well fixed, highly transparent, sharp image.

Background Art

Heat roller fixing type electrostatically charged image developing copiers and printers are gaining popularity because of widespread office automation. With this background, demand is growing for high grade or sharp copied or printed images which are highly light transmissive and well fixed. General formulations for toners in heat roller fixing type electrostatically charged image developing copiers and printers are shown in Table 1. One of the main factors for improving the sharpness, light transmission and strength of fixing of the image is a binder resin, a chief component of the toner. That is, a heat roller fixing type electrostatically charged image developing copier or printer feeds a toner to an electrostatically charged image on a latent image carrier to obtain a visible image, then transfers the resulting toner image to a plain paper or an OHP film, and fixes the transferred image. Currently, styrene-acrylate resin and polyester resin are widely used as binder resins. With the former resin, however, the light transmission and clarity of the resulting toner are not fully satisfactory, resulting in difficulty in obtaining a high grade image. The latter resin, on the other hand, imparts sufficient light transmission, but the resin is yellowish in color, thus decreasing clarity.

Table 1

(Unit: wt. %)

| | Binder resin | Coloring agent | Charge control agent | Functioning agent | Magnetic powder | Solvent |
|----|--------------------------------------|----------------|----------------------|-------------------|-----------------|---------|
| 30 | Dry two component system | 50-100 | 0-20 | 0-10 | 0-20 | - |
| 35 | Dry nonmagnetic one component system | 50-100 | 0-20 | 0-10 | 0-20 | - |
| 40 | Dry magnetic one component system | 0-100 | 0-20 | 0-10 | 0-20 | 0-60 |
| 45 | Liquid toner | 15-50 | 0-10 | 0-5 | 0-10 | 50-70 |

55 The present invention has been accomplished in the light of the aforementioned problems. The object of this invention is to provide a toner in a dry two-component toner developer, a dry nonmagnetic one-component toner developer, a dry magnetic one-component toner developer, and a liquid toner developer which toner gives a higher grade copy image, namely, an image excellent in strength of fixing, light transmission and sharpness, in a heat roller fixing type electrostatically charged image developing copier or printer.

Disclosure of the Invention

A first aspect of the present invention is to provide a toner for developing an electrostatically charged image of a heat roller type copier or printer, the toner consisting essentially of a binder resin, a colorant and a charge control agent, wherein the binder resin at least includes a polyolefin resin having a cyclic structure, and a polyolefin resin of a cyclic structure having an intrinsic viscosity (i.v.) of 0.25 dl/g or more, a heat distortion temperature (HDT) by DIN53461-B of 70°C or higher, and a number average molecular weight of 7,500 or more and a weight average molecular weight of 15,000 or more, as measured by GPC, is contained in a proportion of less than 50% by weight based on the entire binder resin.

10 A second aspect of the invention is to provide the toner for developing an electrostatically charged image according to the first aspect of the invention, in which the binder resin consists of 1 to 100 parts by weight of a polyolefin resin having a cyclic structure, and 0 to 99 parts by weight of at least one resin selected from polyester resins, epoxy resins, polyolefin resins, vinyl acetate resins, vinyl acetate copolymer resins, styrene-acrylate resins, and other acrylate resins.

15 A third aspect of the invention is to provide the toner for developing an electrostatically charged image according to the first or second aspect of the invention, in which the polyolefin resin having a cyclic structure has at least one functional group selected from a carboxyl group, a hydroxyl group and an amino group.

20 A fourth aspect of the invention is to provide the toner for developing an electrostatically charged image according to the first, second or third aspect of the invention, in which the polyolefin resin having a cyclic structure is an ionomer, or has a diene-crosslinked structure.

To solve the problems, we, the inventors, have worked out a measure involving the use of a colorless, highly transparent resin. Examples of such a resin are polycarbonates, polyacrylates, polymethacrylates and polystyrenes. These resins, however, are known to be unsatisfactory in terms of the properties required of binder resins, such as fixing strength and heat response characteristic, and to be questionable when used as binder resins. We have conducted extensive studies to correct these drawbacks, and have found that a toner providing a high grade image can be produced by using a colorless, transparent, highly light transmissive polyolefin resin having a cyclic structure, the polyolefin resin containing less than 50% by weight of a high-viscosity resin based on the entire binder resin. This finding has led us to accomplish the present invention. A toner using as a binder resin a polyolefin resin of a cyclic structure satisfying these characteristics is excellent in fixability, heat response characteristic, and light transmission, achieves a high grade, sharp image, and when used as a color toner, can exhibit its features.

30 The present invention will now be described in detail.

The toner for developing an electrostatically charged image of a heat roller type copier or printer according to the present invention consists essentially of a binder resin, in which the binder resin at least includes a polyolefin resin having a cyclic structure, and a polyolefin resin of a cyclic structure having an intrinsic viscosity (i.v.) of 0.25 dl/g or more, a heat distortion temperature (HDT) by DIN53461-B of 70°C or higher, and a number average molecular weight of 7,500 or more and a weight average molecular weight of 15,000 or more, as measured by GPC, is contained in a proportion of less than 50% by weight based on the entire binder resin.

35 The polyolefin resin having a cyclic structure used herein is, for example, a copolymer of an alpha olefin, such as ethylene, propylene or butylene, with an alicyclic compound having a double bond, such as cyclohexene or norbornene, which copolymer is colorless and transparent, and has high light transmittance. This polyolefin having a cyclic structure is a polymer obtained, for instance, by a polymerization method using a metallocene catalyst or a Ziegler catalyst.

40 Preferred as the colorless, transparent, highly light-transmissive polyolefin of a cyclic structure used in the present invention are a low-viscosity resin having a number average molecular weight of 1,000 to 7,500, preferably 3,000 to 7,500, and a weight average molecular weight of 1,000 to 15,000, preferably 4,000 to 15,000, as measured by GPC, an intrinsic viscosity (i.v.) of less than 0.25 dl/g, and a heat distortion temperature (HDT) by DIN53461-B of lower than 70°C, and a high-viscosity resin having a number average molecular weight of 7,500 or more, preferably 7,500 to 50,000, and a weight average molecular weight of 15,000 or more, preferably 15,000 to 100,000, as measured by GPC, an i.v. of 0.25 dl/g or more, and an HDT of 70°C or higher.

45 The low-viscosity polyolefin having a cyclic structure has the above-mentioned number average molecular weight Mn, weight average molecular weight Mw, intrinsic viscosity (i.v.) and heat distortion temperature (HDT). The Mw/Mn ratio, used as a measure of the degree of dispersion of molecular weight distribution, is as small as from 1 to 2.5, namely, a nearly monodisperse state. Thus, a toner having a quick heat response and a high fixing strength, properties required of a toner, can be realised. This polyolefin resin having a cyclic structure according to the present invention is characterized by the following facts: To T745 with a number average molecular weight of 4,000 to be shown later in Table 2, for example, there was added 5% of the azo pigment Permanent Rubin F6B (Hoechst). The mixture was thoroughly dispersed with a kneader, then formed into a sheet by means of a press, and measured for light transmittance using the Macbeth densitometer RD914 (filter SPI red) with visible light of 624 nm. Its light transmittance was 12.0%. Whereas styrene-acrylate resin showed light transmittance of 7.0%, and polyester resin, 15.5%. Hence, the polyolefin resin having a cyclic structure was confirmed to have high transparency even in a pigment-dispersed system, and to be

usable for a color toner as is polyester resin. Measurement by DSC has shown this polyolefin resin to require very low heat of fusion. Thus, this resin can be expected to markedly reduce energy consumption for fixing.

The high-viscosity polyolefin resin having a cyclic structure has the aforesaid properties. Compared with the same resin with a low viscosity, therefore, this resin imparts structural viscosity to the toner, thereby improving offset preventing effect and adhesion to a material to be copied on, such as a paper or film. The low-viscosity polyolefin resin having a cyclic structure, on the other hand, improves the melt flowability of the toner, and satisfies toner characteristics requiring instantaneous melting and solidifying behaviors.

If the amount of the high-viscosity resin used is 50% or more, however, the uniform kneadability of the resin-pigment mixture extremely will decline, deteriorating toner performance. As a result, the toner will become poor in fixability and heat response characteristic, thus resulting in the failure to obtain a high grade, sharp image.

In the present invention, a toner using a binder resin comprising a mixture of other resin with the polyolefin resin having a cyclic structure, which satisfies the foregoing characteristics, also achieves an image of a high grade, i.e., with high fixing strength and sharpness. In this case, it is preferred that the proportions of the polyolefin resin having a cyclic structure and the other resin in the binder resin are to be 1 to 100, preferably 20 to 90, more preferably 50 to 90 parts by weight of the former, and 0 to 99, preferably 10 to 80, more preferably 10 to 50 parts by weight of the latter. If the amount of the former resin is less than 1 part by weight, it becomes difficult to obtain a high grade image.

If a carboxyl group is introduced into the polyolefin resin having a cyclic structure by the melt air oxidation method or modification with maleic anhydride, its compatibility with the other resin and the dispersability of the pigment can be improved. The same improvement can be achieved by introducing a hydroxyl group or an amino group by a known method.

Furthermore, fixability can be improved by copolymerizing the polyolefin resin having a cyclic structure with a diene monomer such as norbornadiene or cyclohexadiene, or by introducing a crosslinking structure into the polyolefin resin of a cyclic structure, which has a carboxyl group introduced therein, by adding a metal such as zinc, copper or calcium.

The toner for developing an electrostatically charged image of a heat roller type copier or printer according to the present invention can be obtained by adding a colorant, a charge control agent, and if desired, a functioning agent, and other additives to the aforementioned binder resin, and performing known methods such as kneading, grinding and sifting. If desired, a flowing agent may be further added.

The colorant may be a known one, such as carbon black, diazo yellow, phthalocyanine blue, quinacridone, carmine 6B, monoazo red or perylene.

Examples of the charge control agent are known ones such as Nigrosine dyes, fatty acid modified Nigrosine dyes, metallized Nigrosine dyes, metallized fatty acid modified Nigrosine dyes, chromium complexes of 3,5-di-tert-butylsalicylic acid, quaternary ammonium salts, triphenylmethane dyes, and azochromium complexes.

If desired, a known functioning agent, preferably, wax with a melting point of 60 to 170°C, may be added to the toner of the present invention in order to enhance the offset preventing properties during fixing by a heat roller. Examples of the wax with this melting point are carnauba wax, montan wax, and glycerol monostearate.

To the toner of the present invention, there may be further added a flowing agent such as colloidal silica, aluminum oxide or titanium oxide, and a lubricant comprising a fatty acid metal salt such as barium stearate, calcium stearate or barium laurate.

The toner of the present invention may be used as a toner for one component developers or two component developers. Moreover, the toner of the present invention may be used as a one component magnetic toner by incorporating a magnetic powder, or may be used as a full color toner.

The present invention will be described in more detail by reference to Examples and Comparative Examples.

(Toner preparation method I)

Dry nonmagnetic one component system and dry two component system:

Five % by weight of a charge control agent (Copy Charge NX, Hoechst), 2.5% by weight of wax (Hoechst Wax E, Hoechst), 0.5% by weight of aerosol silica (HDK-H2000, Wacker Chemie), 5% by weight of magenta pigment (Permanent Rubin F6B, Hoechst), and 87% by weight of a binder resin were mixed, and melt kneaded at 130°C by a two roll mill. Then, the mixture was cooled down to solidification, and crushed, followed by powderizing the particles using a jet mill. The resulting fine particles were sieved or sifted to select particles with an average particle diameter of about 10 micrometers, thereby preparing a toner.

(Toner preparation method II)

Dry magnetic one component system:

Forty % by weight of a magnetic powder (BL100, Titanium Industry), 5% by weight of a charge control agent (Copy Charge NX, Hoechst), 2.5% by weight of wax (Hostastat FE-2, Hoechst), 0.5% by weight of aerosol silica (HDK-H2000,

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Wacker Chemi), 2% by weight of calcium carbonate (Shiraishi Calcium), and 50% by weight of a binder resin were mixed, and melt kneaded at 150°C by a two roll mill. Then, the mixture was cooled down to solidification, and crushed, followed by powderizing the particles using a jet mill. The resulting fine particles were sifted to select particles with an average particle diameter of about 10 micrometers, thereby preparing a toner.

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(Toner preparation method III)

Liquid toner:

10 Forty % by weight of a mixture consisting of 1 part by weight of carbon black (MA-7, Mitsubishi Kagaku) as a colorant, 0.5 part by weight of a charge control agent (Reflex Blue R51, Hoechst), and 98.5 parts by weight of a binder resin was mixed with 60% by weight of an electrolytic solution (Isopar H, Exxon). The mixture was kneaded with a sand mill to prepare a toner.

15 Examples 1 to 27 and Comparative Examples 1 to 6

Using the toner preparation methods and binder resins shown in Table 2 below, toners of Examples 1 to 27 and Comparative Examples 1 through 6 were produced. Table 3 presents the fundamental properties of the polyolefin resins having a cyclic structure used, and the trade names of other resins used.

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Table 2-1

| Ex.or Comp. Ex.No. | Method of Toner preparation | Formulation of binder resin | | | |
|--------------------|-----------------------------|-----------------------------|------|------------|------|
| | | Sample No. | wt.% | Sample No. | wt.% |
| 1 | I | 1 | 87 | - | - |
| 2 | I | 1 | 58 | 2 | 29 |
| 3 | I | 1 | 58 | 7 | 29 |
| 4 | I | 3 | 87 | - | - |
| 5 | I | 3 | 58 | 7 | 29 |
| 6 | I | 5 | 87 | - | - |
| 7 | I | 5 | 58 | 7 | 29 |
| 8 | II | 1 | 50 | - | - |
| 9 | II | 1 | 30 | 2 | 20 |
| 10 | II | 1 | 30 | 7 | 20 |
| 11 | II | 1 | 30 | 8 | 20 |
| 12 | II | 3 | 50 | - | - |
| 13 | II | 3 | 30 | 7 | 20 |
| 14 | II | 3 | 30 | 8 | 20 |
| 15 | II | 5 | 50 | - | - |
| 16 | II | 5 | 30 | 7 | 20 |
| 17 | II | 5 | 30 | 8 | 20 |
| 18 | III | 1 | 39.4 | - | - |
| 19 | III | 1 | 24 | 2 | 15.4 |
| 20 | III | 1 | 24 | 7 | 15.4 |
| 21 | III | 1 | 24 | 8 | 15.4 |
| 22 | III | 3 | 39.4 | - | - |
| 23 | III | 3 | 24 | 7 | 15.4 |
| 24 | III | 3 | 24 | 8 | 15.4 |
| 25 | III | 5 | 39.4 | - | - |
| 26 | III | 5 | 24 | 7 | 15.4 |
| 27 | III | 5 | 24 | 8 | 15.4 |
| Comp.Ex. | | | | | |
| 1 | I | 7 | 87 | - | - |
| 2 | I | 8 | 87 | - | - |
| 3 | II | 7 | 50 | - | - |
| 4 | II | 8 | 50 | - | - |
| 5 | III | 7 | 39.4 | - | - |
| 6 | III | 8 | 39.4 | - | - |

Table 3

| Sample No. | Product | Mw | Mn | i.v. | HDT | D | Tg |
|------------|----------|---|-------|-------|-----|-----|----|
| 1 | T745 | 7000 | 3800 | 0.19 | <70 | 1.8 | 68 |
| 2 | S-8007 | 70000 | 35000 | 0.8 | ≥70 | 2.0 | 80 |
| 3 | T-745-MO | 6800 | 3400 | <0.25 | <70 | 2.0 | 78 |
| 5 | T-745-CL | 12000 | 3400 | <0.25 | <70 | 3.5 | 76 |
| 10 | 7 | Tafton NE2155 Polyester resin of Kao Corp. | | | | | |
| 15 | 8 | MC100 Styrene-acrylate resin of Nihon Carbide | | | | | |

15 (Evaluations)

The toners prepared by the above toner preparation method I or II were each placed in a commercially available electrophotographic copier (PC100, Canon Inc.), and subjected to performance test. Then, the toners prepared by the toner preparation method III were each placed in a commercially available electrophotographic copier (FT400i, Ricoh Co., Ltd.), and subjected to performance test. The results are shown in Table 4.

Table 4-1

| | Fixability 10 copies per min | Image sharpness | | Light transmission 624 nm | Anti-toner spent properties |
|----|------------------------------|---------------------------|------------|---------------------------|-----------------------------|
| | | Thin line resolving power | Gray scale | | |
| 25 | Ex.1 | ○ | ○ | ○ | ○ |
| 30 | Ex.2 | ○ | ○ | ○ | ○ |
| 35 | Ex.3 | ○ | △ | △ | △ |
| 40 | Ex.4 | ○ | ○ | ○ | ○ |
| 45 | Ex.5 | ○ | ○ | ○ | ○ |
| 50 | Ex.6 | ○ | ○ | ○ | ○ |
| 55 | Ex.7 | ○ | △ | △ | △ |
| | Ex.8 | ○ | ○ | - | ○ |
| | Ex.9 | ○ | ○ | - | ○ |
| | Ex.10 | ○ | ○ | - | △ |
| | Ex.11 | ○ | ○ | - | △ |
| | Ex.12 | ○ | ○ | - | ○ |
| | Ex.13 | ○ | ○ | - | ○ |
| | Ex.14 | ○ | ○ | - | ○ |
| | Ex.15 | ○ | ○ | - | ○ |
| | Ex.16 | ○ | ○ | - | △ |
| | Ex.17 | ○ | ○ | - | △ |
| | Ex.18 | ○ | ○ | - | ○ |
| | Ex.19 | ○ | ○ | - | ○ |
| | Ex.20 | ○ | ○ | - | - |

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Table 4-1 (continued)

| | Fixability 10 copies per min | Image sharpness | | Light transmission 624 nm | Anti-toner spent properties |
|------------|------------------------------|---------------------------|------------|---------------------------|-----------------------------|
| | | Thin line resolving power | Gray scale | | |
| 5 | | | | | |
| Ex.21 | ○ | ○ | ○ | - | - |
| Ex.22 | ○ | ○ | ○ | - | - |
| 10 | Ex.23 | ○ | ○ | - | - |
| Ex.24 | ○ | ○ | ○ | - | - |
| Ex.25 | ○ | ○ | ○ | - | - |
| 15 | Ex.26 | ○ | ○ | - | - |
| Ex.27 | ○ | ○ | ○ | - | - |
| 20 | Comp. Ex.1 | X | △ | △ ○ | X |
| Comp. Ex.2 | X | X | X | X | X |
| 25 | Comp. Ex.3 | X | ○ | ○ - | X |
| Comp. Ex.4 | X | ○ | ○ | - | X |
| Comp. Ex.5 | X | ○ | ○ | - | X |
| Comp. Ex.6 | X | ○ | ○ | - | X |

Evaluation methods and evaluation criteria

1) Fixability

The toners prepared with the respective formulations were each used for copying onto recycled papers at a copying rate of 10 copies/min at a fixing temperature of 110 to 140 °C, with the fixing temperature for each copying cycle being raised by 10 °C. The resulting copy samples were rubbed 10 times with an eraser by using an abrasion tester of Southland. The load during the test was 40 g/cm². The tested samples were measured for the printing density using a Macbeth reflection densitometer. The symbol X was assigned when even one of the measured values at the respective temperatures was less than 65%. The symbol △ was assigned when the measured values at the respective temperatures were 65% or more but less than 75%. The symbol ○ was assigned when the measured values at the respective temperatures were 75% or more.

2) Image sharpness

The toners prepared with the respective formulations were each used for copying onto recycled papers. The resulting samples were checked against sample images of Data Quest. The thin line resolving power and gray scale of the copy image were used as bases for evaluation. The symbol X was assigned when the thin line resolving power was 200 dots/inch or less, △ for a thin line resolving power of 201 to 300 dots/inch, and ○ for a thin line resolving power of 301 dots/inch or more. The ratio of the reflection density of the copy image to that of the reflection density of the sample image, at each step of the gray scale, was evaluated as X when less than 65%, △ when 65% or more but less than 75%, and ○ when 75% or more.

3) Light transmission

The magenta-colored toners prepared with the formulations of the Examples and the Comparative Examples were each used to produce sheet-shaped samples 100 micrometers thick. The light transmission of each sheet sample was measured using an optical filter having a peak at 624 nm. The light transmittance at 624 nm was evaluated as X when less than 8%, △ when 8% or more but less than 11%, and ○ when 11% or more.

4)Anti-toner spent properties

The toner described in each of the Examples and the Comparative Examples and a ferrite carrier of Powdertech were put in predetermined amounts into a developer box. After the mixture was stirred and triboelectrically treated for

5 1 week, 5 g of the toner-deposited carrier was weighed. This carrier was put in water with soap to remove the toner electrostatically adhering to the surface. Only the carrier magnetic powder was collected using a magnet. The magnetic powder was immersed in acetone to dissolve and remove the spent toner fused to the surface. A change in the weight after immersion compared with the weight before immersion was evaluated as ○ when less than 0.2%, △ when 0.2 or more but less than 0.5%, and X when 0.5% or more.

10 The toner for developing an electrostatically charged image of a heat roller type copier or printer according to the present invention contains a binder resin at least including a polyolefin resin having a cyclic structure, in which a high-viscosity polyolefin resin having a cyclic structure is contained in a proportion of less than 50% by weight based on the entire binder resin. Thus, the toner is excellent in fixability, light transmission, and antitonner spent properties, gives a high quality sharp image, and exhibits its features particularly when used in a color toner.

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Claims

1. A toner for developing an electrostatically charged image of a heat roller type copier or printer, said toner consisting essentially of a binder resin, a colorant and a charge control agent, wherein said binder resin at least includes a polyolefin resin having a cyclic structure, and a polyolefin resin of a cyclic structure having an intrinsic viscosity (i.v.) of 0.25 dl/g or more, a heat distortion temperature (HDT) by DIN53461-B of 70 °C or higher, and a number average molecular weight of 7,500 or more and a weight average molecular weight of 15,000 or more, as measured by GPC, is contained in a proportion of less than 50% by weight based on the entire binder resin.
2. The toner for developing an electrostatically charged image as claimed in claim 1, wherein said binder resin consists of 1 to 100 parts by weight of a polyolefin resin having a cyclic structure, and 0 to 99 parts by weight of at least one resin selected from polyester resins, epoxy resins, polyolefin resins, vinyl acetate resins, vinyl acetate copolymer resins, styrene-acrylate resins, and other acrylate resins.
3. The toner for developing an electrostatically charged image as claimed in claim 1 or 2, wherein said polyolefin resin having a cyclic structure has at least one functional group selected from a carboxyl group, a hydroxyl group and an amino group.
4. The toner for developing an electrostatically charged image as claimed in claim 1, 2 or 3, wherein said polyolefin resin having a cyclic structure has a structure crosslinked by metal ions or dienes.

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| INTERNATIONAL SEARCH REPORT | | International application No. PCT/JP96/02133 |
|--|---|---|
| A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁶ G03G9/087 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED <small>Minimum documentation searched (classification system followed by classification symbols)</small> Int. Cl ⁶ G03G9/08, G03G9/087, G03G9/10, G03G9/113 | | |
| <small>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</small> Jitsuyo Shinan Koho 1922 - 1996 Jitsuyo Shinan Toroku Kokai Jitsuyo Shinan Koho 1971 - 1996 Koho 1996 - 1996 Toroku Jitsuyo Shinan Koho 1994 - 1996 | | |
| <small>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)</small> | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | JP, 06-27714, A (Xerox Corp.), February 4, 1994 (04. 02. 94) & US, 5324616, A | 1 - 4 |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. | | <input type="checkbox"/> See patent family annex. |
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| Date of the actual completion of the international search October 9, 1996 (09. 10. 96) | Date of mailing of the international search report October 22, 1996 (22. 10. 96) | |
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(54) ENHANCED LAK CELL ACTIVATION BY TREATMENT OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS WITH AMINO ACID AMIDES.

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- (73) Proprietor: TERUMO KABUSHIKI KAISHA
No. 44-1, Hatagaya 2-chome,
Shibuya-ku
Tokyo 151 (JP)
- (72) Inventor: IRR, Joseph, David
386 Briar Lane
Newark, DE 19711 (US)
Inventor: LEUNG, Kam
709 Marshall Road
Brookhaven, PA 19015 (US)
- (74) Representative: von Kreisler, Alek,
Dipl.-Chem. et al
Patentanwälte
von Kreisler-Selting-Werner
Postfach 10 22 41
D-50462 Köln (DE)

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Description**FIELD OF THE INVENTION**

- 5 This invention relates to the generation of cells having enhanced lymphokine-activated killer (LAK) cell activity which are useful in adoptive immunotherapy.

BACKGROUND OF THE INVENTION

10 Incubation of interleukin-2 (IL-2) with human peripheral blood mononuclear cells (PBMC) or mouse splenocytes induces a population of highly tumoricidal cells. This phenomenon has been referred to as lymphokine-activated killer (LAK) cell activity. The precursor of the LAK effector cells may be heterogeneous, but most of the activity apparently originates from large granular lymphocytes (LGL) which comprise about 5% of peripheral blood lymphocytes (PBL) and which have natural killer (NK) cell activity.

15 Adoptive transfer of LAK cells to tumor-bearing mice, with simultaneous administration of IL-2, has resulted in reduction in tumor burden in several animal models. From these results clinical trials were developed utilizing LAK cells alone, IL-2 alone, and finally an intensive treatment program utilizing both agents in patients with advanced solid tumors (Rosenberg et al., (1987) *N. Engl. J. Med.* 316:889-897; U.S. Patent 4,690,915 issued to Rosenberg). The toxicity of this combination regimen was considerable despite 20 the fact that Rosenberg was able to deliver in man only 1 to 10% of the equivalent dose compared to the effective murine doses of LAK cells and IL-2 (based on weight). Nevertheless, a significant number of partial responses were seen and further trials of the combination of IL-2 and LAK cells are underway.

Central to the problem of the utilization of LAK cells in man is the complexity of their generation (also referred to herein as induction or activation): patients are leukapheresed, the leukapheresis product is 25 separated by Ficoll-Hypaque gradients, and the resultant mononuclear cells are then cultured in the presence of IL-2 at cell densities of 1 to 3×10^6 cells per mL for 3 to 5 days. Thus, the final culture volumes in roller bottles can reach 40 L. Various changes and improvements have been made in this procedure. For example, European patent application EP-A-0247613, published December 2, 1987, and co-assigned, allowed U.S. patent application US-A-4849329 filed April 20, 1987, disclose that depletion of monocytes by 30 exposure of mononuclear cells to phenylalanine methyl ester (PME) allows LAK cell induction at cell culture densities about 10-fold greater than the currently utilized LAK cell induction concentrations. European patent application EP-A-0280054 published August 31, 1988 disclose that LAK cell induction can be carried out in bags made of organic polymeric, oxygen permeable film. European patent application EP-A-0289896 published November 9, 1988, and co-assigned U.S. Patent 4,808,151, issued February 28, 1989, disclose 35 that the leukapheresis product need not be separated on a Ficoll-Hypaque gradient prior to use in the activation process.

Incubating mixtures of human white blood cells collected from the peripheral blood by venipuncture or leukapheresis with esters of various amino acids leads to the transient or permanent loss of functional natural killer (NK) cells (Thiele et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:2468-2472; U.S. Patent 4,752,602 40 issued to Lipsky and Theile). With the methyl ester of leucine (LME), a dipeptide leucyleucine methyl ester is formed in cells exposed to LME, which is toxic to the NK cells (Thiele et al. (1985) *Proc. Acad. Sci. USA* 82:2468-2472; U.S. Patent 4,752,602). The use of PME and other lower alkyl amino acid esters, including the esters of alanine, aspartic acid, cysteine, glutamic acid, glutamine, phenylalanine, proline, tyrosine, tryptophan, and valine, or a mixture of any of the foregoing, in a process to prepare LAK cells is disclosed 45 in European patent application EP-A-0247613, published December 2, 1987, and allowed U.S. application US-A-4849329 filed April 20, 1987.

A problem still exists with the processes described in the art due to the complexity and high volume of the induction or activation systems needed for human LAK cell immunotherapy. The present application describes a process for LAK cell activation at high cell density therefore rendering murine equivalent doses 50 of LAK cell therapy in humans as a feasible alternative.

SUMMARY OF THE INVENTION

This invention relates to enhanced lymphokine-activated killer (LAK) cell activation and more particularly 55 to an improved process for LAK cell activation. In the process wherein peripheral blood mononuclear cells (PBMC) are cultured to produce a population of cells which are cytotoxic for fresh tumor cells, the improvement comprises contacting the PBMC or peripheral blood lymphocytes (PBL) resulting therefrom, prior to culturing said cells at high density, with an L-amino acid amide, wherein the L-amino acid is

s lect d from the group consisting of leucine, isoleucine, phenylalanine, and valine, or a mixture of any of th for going, and thereafter culturing the resulting cells.

Furthermor , the present invention relates to improved processes described above, wherein an L-amino acid amide is used in combination with an L-amino acid lower alkyl ester.

This application describes the use of L-amino acid (aa) amides and preferably L-phenylalaninamide (PAA) for the removal of monocytes, prior to the incubation of the lymphocytes with IL-2 for the production of LAK cells in high cell density cultures. As used herein "high cell density" means cell densities where there is an inhibition of LAK cell production with respect to that obtained in a culture of lower cell density; "high cell density" means cell density greater than 3×10^6 cells/ml to 1×10^8 cells/ml and preferably 1×10^7 cells/ml. For PBMC from certain donors, PAA appears to enhance the extent of LAK cell activation at high cell density compared to the activation obtained by treatment with L-phenylalanine methyl ester (PME) of the same batch of human cells.

Thus, we have developed a practical process to augment the number of LAK cells available for human LAK cell therapy and simultaneously reduce the complexity and volume of the induction system, so that murine equivalent doses of LAK cell therapy are now feasible in man.

The effect of aa amides on LAK cell generation at high cell density does not correlate with the effect of the corresponding aa alkyl ester. For example, the L-amino acid lower alkyl ester of aspartic acid, glutamic acid, and tyrosine enhances generation of LAK cells at high cell density (European patent application EP-A-0247613, published December 2, 1987 and allowed U.S. patent application US-A-4849329 filed April 20, 1987), whereas the corresponding aa amide of these amino acids did not function to enhance LAK cell generation (Example 5, Table 8). Moreover, some aa alkyl esters, including leucine and isoleucine methyl ester, do not enhance LAK cell generation at high cell density (European patent application EP-A-0247613, published December 2, 1987 and allowed U.S. patent application US-A-4849329 filed April 20, 1987), whereas the corresponding aa amides of these amino acids were found to enhance LAK cell production when the cells are cultured at high cell density (Example 5, Table 8).

Although all of the 20 possible amino acid amides have not been tested, it is expected that some of these aa amides, in addition to leucine, isoleucine, phenylalanine, and valine will be useful for enhancing the production of LAK cells when the cells are cultured at high cell density.

30 DETAILED DESCRIPTION OF THE INVENTION

LAK cell activation can be achieved by culturing lymphocytes from the peripheral blood with IL-2 or recombinant IL-2 (rIL-2) if the cell concentration is limited to 3×10^6 /ml, even when there are numerous red blood cells (RBC) present, as disclosed by Dunn, Halpern and Irr in co-assigned U.S. Patent 4,808,151, issued February 28, 1989. When the monocytes are removed from such a preparation of mononuclear cells by chemical or physical means, such as with PME treatment or adherence to surfaces, and the RBC are removed by gradient separation, then the remaining lymphocytes may be cultured at densities of at least 1×10^7 /ml (European patent application EP-A-0247613, published December 2, 1987 and allowed U.S. application US-A-4849329 filed April 20, 1987).

In the process of this invention the PBMC are exposed to a salt of an L-amino acid amide at about room temperature for 40 minutes in a phosphate buffered saline solution, pH 7.0 (PBS), containing 0.5% human serum albumin (Albuminar-25, Armour Pharmaceutical Co., Kankakee, IL). The optimum duration of treatment and temperature at which the cells are treated may be varied and will depend, for example, on the concentration of aa amide used. Other buffered solutions compatible with cell viability may also be used. This treatment results in destruction of monocytes present in the PBMC mixture. The remaining lymphocytes from the treated PBMC are isolated by Ficoll-Hypaque density gradient separation. The isolated cells are washed with PBS and then resuspended in a suitable cell culture medium, such as AIM-V (available from Gibco, Inc., Grand Island, NY). Other suitable media are well known and may be used in place of AIM-V, and are optionally supplemented with human or fetal calf serum for subsequent LAK cell activation.

A preferred embodiment of this invention provides for exposing the PBMC to the hydrochloride salt of L-phenylalaninamide (PAA) at room temperature for 40 minutes in PBS, pH 7.0 containing 0.5% human serum albumin. The PAA may be present in a conc ntration of 1 to 10 mM and pr ferably is pr sent in a concentration of 10 mM.

A further embodiment of this invention provides for exposing the PBMC to a combination of an L-amino acid amide and an L-amino acid lower alkyl ester. Preferably th combination used is PAA pr sent in a concentration of 1 to 10 mM and PME pr s nt in a concentration of 1 to 5 mM.

Once the isolated lymphocytes are counted by standard laboratory methods, for example, using a hemacytometer and microscope, they are diluted to the desired concentration with AIM-V medium and placed in an appropriate cell culture container to be cultured at high densities. The preferred container is the SteriCell™ container, a gas permeable plastic bag commercially available from E. I. du Pont de Nemours & Co., Wilmington, DE. However, other gas permeable bags known in the art may be used. To achieve LAK cell activation, the cells are incubated with rIL-2 for three days at 37°C, 5% CO₂ and 95% relative humidity. Following the three days of incubation, LAK cell activity may be evaluated by testing portions of the cells in a cytotoxicity assay.

10 Cytotoxicity Assay

In the following examples, a 4-hour ⁵¹Cr release assay was used to measure cytotoxic activity of LAK cell preparations. The targets used (Raji and Daudi cell lines) are human tumor cell lines well known for their relative insensitivity to NK cell-mediated cytotoxicity, but susceptibility to LAK cell-mediated cytotoxicity. The tumor target cells at a concentration of 2x10⁶ to 1x10⁷ per ml were incubated with 100 µCi of Na₂⁵¹CrO₄ in 0.4 ml of Tris-phosphate buffered saline for 1 hour at 37°C. The cells were washed three times with RPMI-1640 cell culture medium (Whittaker Bioproducts, Walkersville, MD) containing 10% fetal calf serum (FCS), and resuspended to 10⁵ cells per ml in RPMI-10% FCS. The effector cells (LAK cells) were resuspended to various concentrations in RPMI-10% FCS and 0.1 ml portions were placed into round bottom wells in microtiter plates. The ⁵¹Cr-labeled tumor target cells (0.1 ml) were added to all wells. After 4 hours incubation at 37°C, the plates were centrifuged and 0.1 ml of resulting supernatant fluid was removed from each well and counted in a gamma counter results are expressed in counts per minute (CPM). Each sample of LAK cells was tested in triplicate and the resulting data are expressed as % cytotoxicity.

25 Percent cytosis is calculated from the following formula:

$$\frac{\text{experimental CPM} - \text{spontaneous CPM}}{\text{total CPM} - \text{spontaneous CPM}} \times 100$$

This cytotoxicity test is widely used and is further described in Selected Methods in Cellular Immunology, Mishell and Shiigi, eds. 124-137, W. H. Freeman and Co., San Francisco, CA (1980). In some of the experiments the results of the assays are presented as lytic units (LU or LU₃₀). One lytic unit is defined as 10⁶ divided by the number of effector cells required to lyse 30% of the target cells. This value is computed from fitting a model wherein the % cytosis is a function of the logarithm of the effector:target ratio. This calculation is based upon the method described by Pross et al., J. of Immunological Methods 68:35-249 (1984).

40 Cells: Human peripheral blood cells were collected from healthy donors on the Haemonetics V50 instrument by means of standard cytapheresis protocols as described in U.S. Patents 4,464,167 and 4,416,654. Raji and Daudi cell lines were maintained in continuous culture by standard laboratory procedures; these cell lines were used as the tumor targets in the ⁵¹Cr release assays.

45 Materials:

A. Media: The culture medium used for LAK cell activation consisted of AIM-V™ serum free medium with L-glutamine, streptomycin sulfate and gentamicin sulfate (Gibco). The medium used for culture of cell lines and ⁵¹Cr release assays was RPMI-1640 with L-glutamine (Gibco) and 10% heat inactivated fetal bovine serum and 0.05 µg/ml gentamicin sulfate.

50 Dulbecco's Phosphate-Buffered Saline without Ca++ , Mg++ or Phenol red (PBS) (Gibco) was used for monocyte depletion with L-aa amides and/or L-aa lower alkyl esters and also for cell washing.

B. 50 mM PME Reagent: One gram of L-phenylalanine methyl ester, HCl (Du Pont) was added to 100 ml of PBS and 2 ml of human albumin (Albuminar®-25 U. S. P. 25%). The pH was adjusted to 7.0 with 0.1 N NaOH (Sigma).

C. 50 mM PAA R agent: One gram of L-phenylalaninamide, HCl (Sigma) was added to 100 ml PBS and 2 ml of Albuminar®-25 U.S.P 25%. The pH was adjusted to 7.0 with 0.1 N NaOH.

Other materials used are the same as those described in co-assigned U.S. Patent 4,808,151. Procedure:

- A. Collection of donor cells were by leukapheresis, using methods identical to those described in co-assigned U.S. Patent 4,808,151.
- 5 B. Before further processing a sample of the leukapheresis product was removed for analysis. The remainder was placed in a centrifuge and spun at 468xg for 10 minutes for plasma and platelet separation and removal. The cell fraction was counted for white blood cells (WBC) and adjusted to a density of $1 \times 10^7/\text{mL}$ with PBS supplemented with 0.5% human serum albumin. Portions of the cell suspension were treated with various concentrations of PAA from 1 to 10 mM or 5 mM PME for 40 minutes at room temperature and then were fractionated by the lymphocyte separation procedure.
- 10 C. Lymphocyte Separation: Forty mL of the blood cell suspension were underlaid with 10 mL of lymphocyte separation media (Ficoll-Paque, Pharmacia Fine Chemicals or Lymphoprep, Nycomed). The mixture was then centrifuged for 15 minutes at 800xg. After centrifugation, the resulting interface layer of mononuclear cells was collected and washed twice with PBS. The cells were washed a third time with AIM-V medium and a WBC count was performed.
- 15 D. Culturing Cells in Du Pont Stericell™ Containers. Cells were diluted to a density of $1 \times 10^7/\text{mL}$ in AIM-V medium. Du Pont rIL-2, 100 units/mL, was added and the cultures were transferred into Stericell™ containers. These bags of cells were placed in a 37°C incubator with 5% CO₂ and 95% RH for 3 or 4 days.
- 20 E. In vitro cytotoxicity assay.
- Target cell preparation. The day preceding the assay, exponentially growing target cells were diluted to $1 \times 10^5/\text{mL}$ in 10 mL of assay medium. On the assay day, 4×10^6 cells were washed and resuspended in 0.1 mL 2xTD buffer and 100 µCi ⁵¹CrO₄. The cells were incubated one hour at 37°C, washed twice, counted and diluted to $1 \times 10^5/\text{mL}$ in assay medium.
- 25 Effector cell preparation. About 10 mL of the effector cell culture was washed twice with assay medium, counted and tested for viability. The cells were diluted to $4 \times 10^6/\text{mL}$ in assay medium. Effectors and targets were mixed at ratios of 40:1, 20:1, 10:1, 5:1 and 2.5:1 in 96 well microtiter plates where they were incubated at 37°C for 4 hours before the supernatant fractions were analyzed for ⁵¹Cr content in a gamma counter.

EXAMPLES

Example 1

The effectiveness of L-phenylalaninamide, HCl (PAA) on human monocyte depletion and LAK cell activation in high cell density cultures was examined. PAA and other amino acid amides were obtained from Sigma, St. Louis, MO. For comparative purposes L-phenylalanine methyl ester, HCl (PME) treatments were also performed with aliquots of cells from each preparation of freshly donated human cells.

Sufficient cells were obtained from a donor (designated donor 1) to compare monocyte depletion and high density LAK cell activation in two preparations, one of which was treated with 5 mM PAA, and the other with 5 mM PME. Monocyte depletion was studied by standard hematological differential staining (Table 1A) and by FACS analysis (Table 1B). Smears or cytocentrifuge smears were exposed to Giemsa stain before they were evaluated by light microscopy. A fluorescent-activated cell sorter (FACS) and a panel of conjugated antibodies specific for human cell surface markers were used to quantify the various cell types; these antibodies, Leu4, Leu12, LeuM3, and Leu19, were obtained from Becton Dickinson, Mountain View, CA. As shown, the samples treated with 5 mM PAA and 5 mM PME lost existing monocytes and were, thereby, enriched for lymphocytes.

Table 1A

| Donor 1: Differential Staining | | | |
|--------------------------------|-------------|-----------|--------------|
| Sample | Lymphocytes | Monocytes | Granulocytes |
| Untreated | 67 | 32 | 1 |
| 5 mM PAA Treated | 99 | 1 | 0 |
| 5 mM PME Treated | 91 | 9 | 0 |

Table 1B

| Donor 1: FACS Analysis | | | | |
|------------------------|-------|--------|--------|--------|
| Sample | Leu4* | Leu12* | LeuM3* | Leu19* |
| Untreated | 61 | 10 | 27 | 11 |
| 5 mM PAA Treated | 81 | 7 | 1 | 9 |
| 5 mM PME Treated | 80 | 13 | 5 | 9 |

Leu4* = T Lymphocytes
Leu12* = B Lymphocytes
LeuM3* = Monocytes
Leu19* = NK cells

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The PAA- and PME-treated cells were assayed for LAK cell activity against the two tumor cell targets, Raji and Daudi, following three days of culture in 48 ml of rIL-2-supplemented AIM-V medium in SteriCell™ containers (Table 1C). The overall yields of cells from both treatments yielded greater than 100% recovery from the cultures. These cytolytic activities show that for this particular donor cell preparation greater LAK cell activation was obtained with cells exposed to 5.0 mM PAA than in those treated with 5.0 mM PME.

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Table 1C

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| Donor 1: LAK Activity | | | | | |
|-----------------------|-----------|-------------|-------|------|-------|
| Sample | E:T Ratio | % Cytolysis | | LU30 | |
| | | Raji | Daudi | Raji | Daudi |
| 5 mM PAA | 40:1 | 54.6 | 67.2 | 13.8 | 15.8 |
| | 20:1 | 46.6 | 46.9 | | |
| | 10:1 | 32.8 | 34.5 | | |
| | 5:1 | 26.9 | 28.2 | | |
| | 2.5:1 | 13.1 | 14.4 | | |
| 5 mM PME | 40:1 | 44.9 | 60.7 | 9.3 | 12.7 |
| | 20:1 | 34.6 | 48.3 | | |
| | 10:1 | 34.1 | 28.3 | | |
| | 5:1 | 22.3 | 19.7 | | |
| | 2.5:1 | 9.4 | 13.9 | | |

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Example 2

45 The effects of varied concentrations of PAA on the activation of human LAK cell in high density culture was examined. Again, 5 mM PME treatment of an aliquot of the cells was included as an internal control of the study.

50 The cells from a leukapheresis collection from donor 2, designated donor 2 cells, were divided into six portions for treatment with PAA at various concentrations, a 5 mM PME control and an untreated control. Differentials and FACS analyses were run on specimens after treatment and the remaining cells were cultured for LAK cell activation, as with donor 1 cells (Example 1). The results obtained using donor 2 cells are given in Tables 2A, 2B, and 2C. The extent of monocyte depletion was similar in all of the chemically treated samples. The extent of LAK cell activation was related to the concentration of PAA used. As shown in Table 2C, the optimum PAA concentration for LAK generation was 10 mM and PAA appeared to be somewhat more effective than 5 mM PME for LAK cell generation, for cells from donor 2. 5 mM is the known optimum concentration for PME (see, for example, Table 6).

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Table 2A

| Donor 2: Differential Staining | | | |
|--------------------------------|-------------|-----------|--------------|
| Sample | Lymphocytes | Monocytes | Granulocytes |
| Untreated | 76 | 22 | 2 |
| 5 mM PAA Treated | 90 | 9 | 1 |
| 10 mM PAA Treated | 90 | 6 | 4 |
| 15 mM PAA Treated | 91 | 5 | 4 |
| 20 mM PAA Treated | 87 | 8 | 7 |
| 5 mM PME Treated | 95 | 4 | 1 |

Table 2B

| Donor 2: FACS Analysis | | | | |
|------------------------|-------|--------|--------|--------|
| SAMPLE | Leu4* | Leu12* | LeuM3* | Leu19* |
| Untreated | 75 | 10 | 18 | 9 |
| 5 mM PAA Treated | 73 | 6 | 8 | 5 |
| 10 mM PAA Treated | 76 | 8 | 6 | 5 |
| 15 mM PAA Treated | 77 | 8 | 5 | 5 |
| 20 mM PAA Treated | 78 | 7 | 5 | 4 |
| 5 mM PME Treated | 75 | 7 | 7 | 6 |

Leu4* = T Lymphocytes
 Leu12* = B Lymphocytes
 LeuM3* = Monocytes
 Leu19* = NK cells

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Table 2C

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Donor 2: LAK Activity

| | <u>Sample</u> | <u>E:T Ratio</u> | % Cytolysis | | LU30 | |
|----|---------------|------------------|-------------|-------|------|-------|
| | | | Raji | Daudi | Raji | Daudi |
| 10 | Untreated | 40:1 | 7.7 | 17.6 | | |
| | | 20:1 | 2.8 | 0.7 | | |
| | | 10:1 | 11.3 | 7.9 | 0 | 0 |
| | | 5:1 | 5.0 | 6.7 | | |
| | | 2.5:1 | 2.8 | 5.1 | | |
| | | | | | | |
| 15 | 5 mM PAA | 40:1 | 43.1 | 38.1 | | |
| | | 20:1 | 36.6 | 28.5 | | |
| | | 10:1 | 8.1 | 4.9 | 5.2 | 3.4 |
| | | 5:1 | 7.0 | 4.0 | | |
| | | 2.5:1 | 9.0 | 5.7 | | |
| | | | | | | |
| 20 | 10 mM PAA | 40:1 | 57.9 | 44.4 | | |
| | | 20:1 | 43.9 | 39.2 | | |
| | | 10:1 | 39.3 | 30.4 | 11.5 | 7.6 |
| | | 5:1 | 22.5 | 13.3 | | |
| | | 2.5:1 | 0 | 0 | | |
| | | | | | | |
| 25 | 15 mM PAA | 40:1 | 19.1 | 8.8 | | |
| | | 20:1 | 16.9 | 9.4 | | |
| | | 10:1 | 10.2 | 4.5 | 0.5 | 0 |
| | | 5:1 | 7.0 | 5.2 | | |
| | | 2.5:1 | 2.8 | 4.2 | | |
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Table 2C (cont'd)

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|----|-----------|-------|------|------|-----|-----|
| 5 | | 40:1 | 31.7 | 21.8 | | |
| | | 20:1 | 14.6 | 7.4 | | |
| 10 | 20 mM PAA | 10:1 | 18.6 | 12.6 | 1.6 | 0.2 |
| | | 5:1 | 10.6 | 6.9 | | |
| | | 2.5:1 | 8.9 | 4.4 | | |
| | | | | | | |
| 15 | | 40:1 | 48.0 | 52.6 | | |
| | | 20:1 | 40.6 | 37.9 | | |
| 20 | 5 mM PME | 10:1 | 7.4 | 7.1 | 6.1 | 6.3 |
| | | 5:1 | 5.0 | 4.4 | | |
| | | 2.5:1 | 6.2 | 7.5 | | |

25 Cells from two other donors, designated donors 3 and 4, were used to further examine the effects of varied concentrations of PAA on LAK cell activation. The results obtained using donors 3 and 4 cells are given in Tables 3A, 3B, 3C, 4A, 4B, and 4C. With these cells, lower concentrations of PAA were tested before the high density activation step. In both cases there was a concentration dependent effect on the 30 LAK cell activation response. This dependence upon PAA for efficient LAK cell activation was particularly noticeable in these samples because the untreated cells failed to produce LAK cell activity. The PME-treated cells from donor 4 were not active in the LAK cell assays whereas, PAA did enhance LAK activity in donor 4 cells (Table 4C). The results suggest that, in some donors, a greater level of LAK activity may be obtained using PAA than can be obtained using PME.

35 Table 3A

| Donor 3: Differential Staining | | | | |
|--------------------------------|--------------------|-------------|-----------|--------------|
| | Sample | Lymphocytes | Monocytes | Granulocytes |
| 40 | Untreated | 71 | 28 | 1 |
| | 0.5 mM PAA Treated | 74 | 26 | 0 |
| 45 | 2.5 mM PAA Treated | 86 | 14 | 0 |
| | 5 mM PAA Treated | 92 | 7 | 1 |
| | 10 mM PAA Treated | 95 | 5 | 0 |
| | 5 mM PME Treated | 93 | 4 | 3 |

Table 3B

| Donor 3: FACS Analysis | | | | |
|--|-------|--------|--------|--------|
| SAMPLE | Leu4* | Leu12* | LeuM3* | Leu19* |
| Untreated | 75 | 13 | 18 | 12 |
| 0.5 mM PAA Treated | 80 | 14 | 19 | 7 |
| 2.5 mM PAA Treated | 81 | 12 | 10 | 7 |
| 5 mM PAA Treated | 83 | 15 | 6 | 16 |
| 10 mM PAA Treated | 79 | 13 | 7 | 13 |
| 5 mM PME Treated | 70 | 12 | 6 | 10 |
| Leu4* = T Lymphocytes Leu12* = B Lymphocytes LeuM3* = Monocytes Leu19* = NK cells | | | | |

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Table 3C

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Donor 3: LAK Activity

| | <u>Sample</u> | <u>E:T Ratio</u> | <u>% Cytolysis</u> | | <u>LU30</u> | |
|----|---------------|------------------|--------------------|-------|-------------|-------|
| | | | Raji | Daudi | Raji | Daudi |
| 10 | Untreated | 40:1 | 15.6 | 9.6 | | |
| | | 20:1 | 0 | 1.3 | | |
| | | 10:1 | 8.6 | 4.6 | 0 | 0 |
| | | 5:1 | 0 | 2.6 | | |
| | | 2.5:1 | 0 | 3.3 | | |
| | | | | | | |
| 15 | 0.5 mM PAA | 40:1 | 7.4 | 9.7 | | |
| | | 20:1 | 4.5 | 4.3 | | |
| | | 10:1 | 0 | 0 | 0 | 0 |
| | | 5:1 | 0 | 0 | | |
| | | 2.5:1 | 0 | 0 | | |
| | | | | | | |
| 20 | 2.5 mM PAA | 40:1 | 23.1 | 13.9 | | |
| | | 20:1 | 18.3 | 10.0 | | |
| | | 10:1 | 11.6 | 9.2 | 0.9 | 0.1 |
| | | 5:1 | 3.6 | 4.0 | | |
| | | 2.5:1 | 0 | 0 | | |
| | | | | | | |
| 25 | 5 mM PAA | 40:1 | 31.0 | 30.5 | | |
| | | 20:1 | 27.0 | 24.2 | | |
| | | 10:1 | 16.7 | 15.8 | 1.9 | 2.4 |
| | | 5:1 | 6.6 | 10.2 | | |
| | | 2.5:1 | 3.5 | 7.1 | | |
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Table 3C (Cont'd)

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|----|-----------|-------|------|------|-----|-----|
| 5 | | 40:1 | 38.8 | 44.1 | | |
| | | 20:1 | 23.6 | 24.2 | | |
| 10 | 10 mM PAA | 10:1 | 19.9 | 20.2 | 2.6 | 5.1 |
| | | 5:1 | 14.4 | 12.8 | | |
| | | 2.5:1 | 5.5 | 7.3 | | |
| | | | | | | |
| 15 | | 40:1 | 44.4 | 44.8 | | |
| | | 20:1 | 30.9 | 39.3 | | |
| 20 | 5 mM PME | 10:1 | 7.2 | 11.4 | 3.3 | 6.0 |
| | | 5:1 | 3.5 | 8.2 | | |
| | | 2.5:1 | 3.9 | 6.3 | | |
| | | | | | | |

Table 4A

| Donor 4: Differential Staining | | | | |
|--------------------------------|--------------------|-------------|-----------|--------------|
| | Sample | Lymphocytes | Monocytes | Granulocytes |
| 35 | Untreated | 54 | 43 | 3 |
| | 0.5 mM PAA Treated | 56 | 42 | 2 |
| | 2.5 mM PAA Treated | 73 | 25 | 2 |
| | 5 mM PAA Treated | 85 | 14 | 1 |
| | 10 mM PAA Treated | 83 | 14 | 3 |
| 40 | 5 mM PME Treated | 93 | 6 | 1 |

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Table 4B

| Donor 4: FACS Analysis | | | | |
|------------------------|-------|--------|--------|--------|
| SAMPLE | Leu4* | Leu12* | LeuM3* | Leu19* |
| Untreated | nt | nt | nt | nt |
| 0.5 mM PAA Treated | nt | nt | nt | nt |
| 2.5 mM PAA Treated | 63 | 7 | 18 | 7 |
| 5 mM PAA Treated | 62 | 8 | 11 | 12 |
| 10 mM PAA Treated | 71 | 7 | 5 | 10 |
| 5 mM PME Treated | 65 | 9 | 6 | 7 |

Leu4* = T Lymphocytes
 Leu12* = B Lymphocytes
 LeuM3* = Monocytes
 Leu19* = NK cells
 nt = not tested

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Table 4C

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Donor 4: LAK ACTIVITY

| 10 | SAMPLE | E:T RATIO | % CYTOLYSIS | | LU30 | |
|----|------------|-----------|-------------|-------|------|-------|
| | | | RAJI | DAUDI | RAJI | DAUDI |
| 15 | UNTREATED | 40:1 | 0 | 7.2 | | |
| | | 20:1 | 0 | 1.0 | | |
| | | 10:1 | 0 | 1.2 | 0 | 0 |
| | | 5:1 | 0 | 0 | | |
| | | 2.5:1 | 0 | 0 | | |
| | | | | | | |
| 20 | 0.5 mM PAA | 40:1 | 0 | 9.6 | | |
| | | 20:1 | 0 | 5.8 | | |
| | | 10:1 | 0 | 0 | 0 | 0 |
| | | 5:1 | 0 | 0 | | |
| | | 2.5:1 | 0 | 0 | | |
| | | | | | | |
| 25 | 2.5 mM PAA | 40:1 | 4.2 | 8.3 | | |
| | | 20:1 | 0 | 2.7 | | |
| | | 10:1 | 0 | 6.2 | 0 | 0 |
| | | 5:1 | 0 | 0 | | |
| | | 2.5:1 | 0 | 0 | | |
| | | | | | | |
| 30 | 5 mM PAA | 40:1 | 17.4 | 21.9 | | |
| | | 20:1 | 14.9 | 16.3 | | |
| | | 10:1 | 4.3 | 8.1 | 0.2 | 0.6 |
| | | 5:1 | 11.5 | 5.2 | | |
| | | 2.5:1 | 1.3 | 3.3 | | |
| | | | | | | |

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TABLE 4C (CONT'D)

| | | | | | | |
|----|-----------|-------|------|------|-----|-----|
| 5 | | 40:1 | 34.8 | 42.2 | | |
| | | 20:1 | 16.9 | 24.3 | | |
| 10 | 10 mM PAA | 10:1 | 18.3 | 20.0 | 2.6 | 4.9 |
| | | 5:1 | 10.3 | 16.3 | | |
| | | 2.5:1 | 5.1 | 6.6 | | |
| 15 | | 40:1 | 11.2 | 17.6 | | |
| | | 20:1 | 7.6 | 10.2 | | |
| 20 | 5 mM PME | 10:1 | 0 | 0.9 | 0 | 0.2 |
| | | 5:1 | 0 | 0 | | |
| | | 2.5:1 | 0 | 1.8 | | |

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Example 3

30 The dependence of LAK cell activation at high cell density on the concentration of PAA used to treat a preparation of human PBMC, collected by leukapheresis, which have an unusually high monocyte count was evaluated. Occasionally healthy donor leukapheresis collections result in high monocyte counts. On the average the count is 15 to 25% of the mononuclear cells. We checked several donor preparations prior to further processing. One preparation, designated donor 5, yielded nearly double the normal count of 35 monocytes. That preparation was then divided and tested for LAK cell activation after exposure to 0, 5, 10, 15, or 20 mM PAA. A control of 5 mM PME was also run (Table 5A). FACS analyses were not available for these samples. As seen in previous experiments, there was excellent reduction in the monocyte counts. 50 ml cultures of the cells in rIL-2-supplemented AIM-V cell culture medium yielded better than 100% of the inoculated cell number. The LAK cell activation again showed a response related to the concentration of 40 PAA used. Optimal LAK activity was obtained in the sample treated at 10 mM PAA (Table 5B).

Table 5A

| 45 | Donor 5: Differential Staining | | | |
|----|--------------------------------|-------------|-----------|--------------|
| | Sample | Lymphocytes | Monocytes | Granulocytes |
| | Untreated | 56 | 43 | 1 |
| | 5 mM PAA Treated | 91 | 7 | 2 |
| 50 | 10 mM PAA Treated | 93 | 3 | 4 |
| | 15 mM PAA Treated | 93 | 5 | 2 |
| | 20 mM PAA Treated | 93 | 4 | 3 |
| | 5 mM PME Treated | 95 | 5 | 0 |

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Table 5B

Donor 5 LAK Activity

| E:T Ratio | Untreated | % Cytolysis of Raji Cells | | | | | |
|-----------|-----------|---------------------------|------|------|------|----------|--|
| | | 5 | 10 | 15 | 20 | 5 mM PME | |
| 40:1 | 20.5 | 64.3 | 86.6 | 52.8 | 38.9 | 67.9 | |
| 20:1 | 10.5 | 42.3 | 70.8 | 54.4 | 24.9 | 42.9 | |
| 10:1 | 46.3 | 23.0 | 33.4 | 41.4 | 33.1 | 24.7 | |
| 5:1 | 45.3 | 22.6 | 16.3 | 20.5 | 30.8 | 23.7 | |
| 2.5:1 | 12.9 | 19.2 | 4.7 | 8.4 | 24.7 | 42.3 | |

Table 5C

Donor 5: LAK Activity

| E:T Ratio | Untreated | % Cytolysis of Raji Cells | | | | | |
|-----------|-----------|---------------------------|------|------|------|----------|--|
| | | 5 | 10 | 15 | 20 | 5 mM PME | |
| 40:1 | 28.1 | 47.9 | 71.9 | 36.3 | 22.4 | 59.8 | |
| 20:1 | 19.5 | 34.4 | 52.6 | 31.7 | 12.1 | 40.7 | |
| 10:1 | 29.4 | 15.9 | 18.7 | 21.8 | 19.5 | 23.2 | |
| 5:1 | 19.2 | 16.3 | 27.6 | 12.9 | 13.7 | 21.8 | |
| 2.5:1 | 14.4 | 15.5 | 3.8 | 3.0 | 10.7 | 21.0 | |

Example 4

PBMC were obtained from Biological Specialty (Lansdale, PA) and separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient sedimentation. PBMC (1×10^7 cells/ml) in RPMI-1640 medium, after Ficoll separation, were incubated with freshly prepared PME, PAA, or combinations thereof at room temperature for 40 min. The stock solutions were adjusted to pH 7.4 before the addition to PBMC. The cells were washed with RPMI-1640 medium. Monocyte depletion from PBMC was assessed by Giemsa staining.

The cells were cultured in polypropylene tubs with medium supplemented with 10% fetal calf serum and 400 U/ml of rIL-2 (BRMP units) at a cell density of 1×10^7 cells/ml, for 4 days at 37°C. After the culture period, the resulting cells were harvested for cytotoxicity assays against ^{51}Cr -labeled Raji (to measure LAK activity) and K562 (to measure NK activity) target cells. All assays were carried out in triplicate in round bottom microtiter plates in a total volume of 0.2 ml. Labeled target cells in 0.1 ml (1×10^4 cells) were added to 0.1 ml of effector cells at various concentrations to obtain appropriate final effector cell:target cell ratios (E:T). The microtiter plates were centrifuged for 3 min at 80 x g and then incubated for 2 hr (NK assay) or 4 hr (LAK assay). Three E:T ratios were assessed. The data shown is with E:T ratio of 20:1 in 2 hr assays for K562 and 4 hr assays for Raji.

PAA was compared with the effects of PME on PBMC to assess their effects on monocyte depletion, NK activity and LAK activation by IL-2. PAA depleted monocytes from PBMC in a dose-dependent manner. The NK activity of PAA- or PME-treated cells was also inhibited before culture (Day 0). The activation of LAK activity by IL-2 was also dependent on the amount of monocytes depleted by PAA or PME. In most of the donors tested, PAA and PME were equally effective at enhancing LAK-cell generation, when used at the optimal concentration (Table 6).

For two of the three donors tested, under the conditions used, the combination of PME and PAA did not show any substantial improvement over the use of 5 mM PME alone (Table 7) although it did show good monocyte depletion and LAK activation. However, for one donor (Exp. 2, Table 7), the combination of PAA and PME appeared to be additive. This result indicates that, at least for some donors, the combination of PAA and PME may result in a greater level of LAK activity than can be obtained using either agent alone.

Each experiment in Tables 6 and 7 ("Exp.") represents a separate set of donor cells.

The results of the foregoing examples demonstrate that monocyte depletion and subsequent LAK cell activation based on pretreating the PBMC with PAA and/or PME is dependent on the donor and as such PAA and/or PME are viable treatment alternatives. If for example, PME is initially used to treat a donor's PBMC and insufficient monocyte depletion results with corresponding insufficient LAK cell activation, the clinician can now turn to PAA or a combination of PAA and PME which may yield sufficient monocyte depletion.

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Table 6

| Effect of PME and PAA on Monocyte Depletion, NK Activity and LAK Activation | | | | | |
|---|-------------|-------------|--------------------|--|------------|
| | Exp. 1 | Day 0 | | Cytolytic Activity Following LAK Activation (Day 3-4) | |
| | | % Monocytes | NK Activity (K562) | NK (K562) | LAK (Raji) |
| 5 | Control | 50 | 15.4 | 47.3±1.8 | 3.4±0.4 |
| | PAA (5mM) | 8 | 10.2 | 50.1±0.7 | 8.1±0.6 |
| | PME (2.5mM) | 8 | 8.2 | 58.0±0.5 | 23.8±2.3 |
| 10 | Exp. 2 | | | | |
| | Control | 33 | | 8.7±0.4 | 16.3±1.3 |
| | PAA (5mM) | 11 | | 40.8±1.3 | 59.3±1.1 |
| 15 | PME (5mM) | 3.5 | | 51.2±0.3 | 60.0±2.3 |
| | Exp. 3 | | | | |
| | Control | 34 | 21.±0.6 | 39.1±1.4 | 18.1±1.0 |
| 20 | PAA (1 mM) | 32 | 19.5±0.5 | 45.5±0.5 | 19.8±0.7 |
| | PAA (5 mM) | 13 | 8.7±0.6 | 52.6±0.0 | 30.0±1.6 |
| | PAA (10 mM) | 8 | 8.1±0.4 | 55.0±1.0 | 36.8±1.5 |
| | PME (5 mM) | 1 | 15.1±0.3 | 57.6±6.8 | 44.4±0.6 |
| | PME (10 mM) | 2 | 6.4±1.1 | 52.8±1.6 | 36.0±1.0 |
| 25 | Exp. 4 | | | | |
| | Control | 49.5 | 45.7±1.6 | 46.4±2.1 | 7.4±0.5 |
| | PAA (1 mM) | 27.5 | 36.7±1.5 | 48.3±0.7 | 10.3±0.2 |
| 30 | PAA (5 mM) | 10.5 | 30.9±1.2 | 49.3±0.9 | 12.7±1.2 |
| | PAA (10 mM) | 3 | 20.8±1.0 | 26.7±1.2 | 10.5±0.4 |
| | PME (5 mM) | 2.5 | 14.4±1.0 | 56.6±1.1 | 33.3±0.3 |
| | Exp. 5 | | | | |
| | Control | 14.5 | 54.2±1.0 | 14.3±0.6 | 40.0±1.6 |
| 35 | PAA (5 mM) | 7 | 41.8±0.7 | 25.6±0.8 | 37.3±0.8 |
| | PAA (10 mM) | 1 | 42.0±1.0 | 27.1±0.6 | 50.7±0.7 |
| | PME (5 mM) | 6.5 | 46.0±1.0 | 26.5±0.7 | 49.5±1.4 |
| | PME (10 mM) | 1 | 33.6±0.6 | 27.6±1.0 | 53.3±0.4 |

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Table 7

Effect of Combination of PME and PAA
on Monocyte Depletion, NK Activity and LAK Activation

Exp. 1

| | | [PAA], (mM) | | | |
|----|-------------|---|------|------|------|
| | | 0 | 1 | 5 | 10 |
| 10 | [PME], (mM) | % Monocytes (Day 0) | | | |
| | 0 | 34 | 32 | 13 | 8 |
| | 5 | 1 | 3 | 3 | 3.5 |
| | 10 | 2 | 2 | 6 | 3 |
| 20 | [PME], (mM) | % Cytotoxicity Against K562 (Day 0) | | | |
| | 0 | 21 | 19.5 | 8.7 | 8.1 |
| | 5 | 15.1 | 13.6 | 13.0 | 11.4 |
| | 10 | 6.4 | 10.4 | 10.3 | 8.1 |
| 25 | [PME], (mM) | LAK Activity Against Raji (% Lysis) (Day 3-4) | | | |
| | 0 | 39.1 | 45.3 | 52.6 | 52.0 |
| | 5 | 57.6 | 59.2 | 61.2 | 48.2 |
| | 10 | 52.8 | 56.1 | 51.2 | 51.2 |

Exp. 2

| | | [PAA], (mM) | | | |
|----|-------------|---|------|------|------|
| | | 0 | 1 | 5 | 10 |
| 30 | [PME], (mM) | % Monocytes (Day 0) | | | |
| | 0 | 49.5 | 27.5 | 10.5 | 3 |
| | 5 | 2.5 | 4.5 | 3 | 4.5 |
| | [PME], (mM) | % Cytotoxicity Against K562 (Day 0) | | | |
| 40 | 0 | 45.7 | 36.7 | 30.9 | 20.8 |
| | 5 | 14.4 | 14.0 | 7.3 | 16.6 |
| 45 | [PME], (mM) | LAK Activity Against Raji (% Lysis) (Day 3-4) | | | |
| | 0 | 7. | 10.3 | 12.7 | 10.5 |
| | 5 | 33.3 | 24.0 | 41.3 | 44.3 |

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| <u>Exp. 3</u> | | <u>[PAA], (mM)</u> | | |
|---------------|--------------------|--|------|------|
| | | 0 | 5 | 10 |
| 5 | <u>[PME], (mM)</u> | <u>% Monocytes (Day 0)</u> | | |
| | 0 | 15 | 7 | 2 |
| | 1 | 7 | 1 | 1.5 |
| 10 | 5 | 1.5 | 0 | 1 |
| 15 | <u>[PME], (mM)</u> | <u>% Cytotoxicity Against K562 (Day 0)</u> | | |
| | 0 | 40.6 | 31.3 | 30.5 |
| | 1 | 45.1 | 39.7 | 43.7 |
| 15 | 5 | 33.5 | 39.9 | 17.8 |
| 20 | <u>[PME], (mM)</u> | <u>LAK Activity Against Raji (% Lysis) (Day 3-4)</u> | | |
| | 0 | 55.9 | 57.0 | 61.2 |
| | 1 | 72.2 | 62.0 | 69.0 |
| 25 | 5 | 61.1 | 67.9 | 58.5 |

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Example 5

PBMC were treated with PME or various amino acid amides for 40 min at room temperature. Monocyte content was determined by FACS analysis using LeuM3 antibody, which is a surface marker for monocytes. NK activity (E:T ratio, 25:1) was measured against K562 cells. LAK activation was determined after the cells were incubated with RPMI-1640 containing 4% human serum, and 400 U/ml rIL-2 (BRMP units) for 3 to 4 days at 1×10^7 cells/ml, using Raji cells as targets. The results (Table 8) show that the amino acid amide of leucine, isoleucine, valine, as well as phenylalanine are effective at enhancing the level of LAK activity relative to that obtained in the absence of treatment with amino acid amide. Each experiment (Exp.) in Table 8 represents a separate set of donor cells.

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Table 8

| | Exp. 1 | Day 0 | | Cytolytic Activity Following LAK Activation | |
|--------|---------------------------|-------------|-------------|---|------------|
| | | % Monocytes | NK Activity | NK (K562) | LAK (Raji) |
| 5 | 0 | 23 | 50 | 15 | 3 |
| | PME , 5 mM | 0 | 5 | 64 | 56 |
| | TyrNH ₂ , 5 mM | 25 | 54 | 18 | 5 |
| | 10 mM | 32 | 70 | 9 | 2 |
| | LeuNH ₂ , 5 mM | 1 | 50 | 54 | 28 |
| | 10 mM | 0 | 47 | 64 | 48 |
| Exp. 2 | | | | | |
| 15 | 0 | 12 | 25 | 46 | 14 |
| | PME , 5 mM | 2 | 15 | 61 | 32 |
| | PheNH ₂ , 5 mM | 2 | 34 | 62 | 35 |
| | LME , 5 mM | 1 | 0 | 4 | 5 |
| | LeuNH ₂ , 5 mM | 0 | 35 | 59 | 27 |
| Exp. 3 | | | | | |
| 20 | 0 | 18 | 54 | 22 | 3 |
| | ValNH ₂ , 5 mM | 16 | 48 | 30 | 10 |
| | GluNH ₂ , 5 mm | 24 | 60 | 19 | 3 |
| | AspNH ₂ , 5 mm | 26 | 61 | 20 | 5 |
| | IleNH ₂ , 5 mm | 6 | 51 | 55 | 17 |
| | PME , 5 mm | 3 | 42 | 67 | 22 |

30 Claims

1. In a process for preparing lymphokine-activated killer cells wherein peripheral blood mononuclear cells are cultured to produce a population of cells which are cytotoxic for natural killer cell-resistant tumor cells, the improvement comprising contacting said peripheral blood mononuclear cells or peripheral blood lymphocytes resulting therefrom with an L-amino acid amide, wherein the L-amino acid is selected for the group consisting of leucine, isoleucine, phenylalanine, and valine, or a mixture of any of the foregoing, and thereafter culturing the resulting cells.
2. A process according to Claim 1 wherein said peripheral blood mononuclear cells are human cells.
3. A process according to Claim 2 wherein the contacting is performed for a period of 40 minutes.
4. A process according to Claim 3 wherein the amino acid is phenylalanine.
- 45 5. A process according to Claim 4 wherein the phenylalanine amide is present in a concentration of 1 to 10 mM.
6. A process according to Claim 5 wherein the phenylalanine amide is present in a concentration of 10 mM.
- 50 7. A process according to Claim 3 wherein the human peripheral blood mononuclear cells or peripheral blood lymphocytes resulting therefrom are cultured in the presence of interleukin-2.
8. A process according to Claim 5 wherein the human peripheral blood mononuclear cells or peripheral blood lymphocytes resulting therefrom are cultured in the presence of interleukin-2.
- 55 9. A process according to Claim 7 wherein the human peripheral blood lymphocytes obtained by contacting with the amino acid amide are washed and resuspended.

10. A process according to Claim 8 wherein the human peripheral blood lymphocytes obtain d by contacting with phenylalanine amide are washed and resuspended.
11. A process according to Claim 9 wherein the resuspended human peripheral blood lymphocytes are cultured for 2 to 4 days in the presence of recombinant interleukin-2.
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12. A process according to Claim 10 wherein the resuspended human peripheral blood lymphocytes are cultured for 2 to 4 days in the presence of recombinant interleukin-2.
13. A process according to Claim 11 wherein the concentration of human peripheral blood lymphocytes is from 3×10^6 cells/ml to 1×10^8 cells/ml.
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14. A process according to Claim 12 wherein the concentration of human peripheral blood lymphocytes is from 3×10^6 cells/ml to 1×10^8 cells/ml.
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15. In a process for preparing lymphokine-activated killer cells wherein peripheral blood mononuclear cells are cultured to produce a population of cells which are cytotoxic for natural killer cell-resistant tumor cells, the improvement comprising contacting said peripheral blood mononuclear cells or peripheral blood lymphocytes resulting therefrom with a combination of an L-amino acid amide and an L-amino acid lower alkyl ester, wherein the L-amino acid amide is selected from the group consisting of leucine, isoleucine, phenylalanine, and valine, and the L-amino acid lower alkyl ester is selected from the group consisting of alanine, aspartic acid, cysteine, glutamic acid, glutamine, phenylalanine, proline, tyrosine, tryptophan, and valine, and thereafter culturing the resulting cells.
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16. A process according to Claim 15 wherein the peripheral blood mononuclear cells are human.
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17. A process according to Claim 16 wherein the contacting is performed for a period of 20 to 40 minutes.
18. A process according to Claim 17 wherein the L-amino acid is phenylalanine.
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19. A process according to Claim 18 wherein the ester is L-phenylalanine methyl ester and the amide is phenylalanine amide.
20. A process according to Claim 19 wherein the ester is present in a concentration of 1-5 mM and the amide is present in a concentration of 1-10 mM.
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21. A process according to Claim 20 wherein the hydrogen chloride salt of the ester and the amide is present.
22. A process according to Claim 19 wherein the human peripheral blood mononuclear cells or peripheral blood lymphocytes are cultured in the presence of interleukin-2.
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23. A process according to Claim 20 wherein the human peripheral blood mononuclear cells or peripheral blood lymphocytes are cultured in the presence of interleukin-2.
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24. A process according to Claim 22 wherein the peripheral blood lymphocytes obtained by contacting with the ester and amide are washed and resuspended.
25. A process according to Claim 23 wherein the peripheral blood lymphocytes obtained by contacting the ester and amide are washed and resuspended.
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26. A process according to Claim 24 wherein the resuspended peripheral blood lymphocytes are cultured for 2-4 days in the presence of recombinant interleukin-2.
27. A process according to Claim 25 wherein the resuspended peripheral blood lymphocytes are cultured for 2-4 days in the presence of recombinant interleukin-2.
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28. A process according to Claim 26 wherein the concentration of peripheral blood lymphocytes is from 3x10⁶ cells/ml to 1x10⁸ cells/ml.
- 5 29. A process according to Claim 27 wherein the concentration of peripheral blood lymphocytes is from 3x10⁶ cells/ml to 1x10⁸ cells/ml.

Patentansprüche

1. Verfahren zur Herstellung von Lymphokin-aktivierten Killerzellen, in dem periphäre einkernige Blutzellen zur Herstellung einer Zellpopulation kultiviert werden, die für Tumorzellen, die gegen natürliche Killerzellen resistent sind, cytotoxisch sind, umfassend das In-Berührung-Bringen der peripheren einkernigen Blutzellen oder der daraus entstehenden peripheren Blut-Lymphocyten mit einem L-Aminosäureamid, wobei das L-Aminosäureamid aus der aus Leucin, Isoleucin, Phenylalanin und Valin oder einer Mischung irgendwelcher der vorgenannten bestehenden Gruppe ausgewählt ist, und danach das Kultivieren der entstandenen Zellen.
2. Verfahren nach Anspruch 1, wobei die peripheren einkernigen Blutzellen menschliche Zellen sind.
3. Verfahren nach Anspruch 2, wobei das In-Berührung-Bringen für einen Zeitraum von 40 Minuten durchgeführt wird.
4. Verfahren nach Anspruch 3, wobei die Aminosäure Phenylalanin ist.
5. Verfahren nach Anspruch 4, wobei das Phenylalaninamid in einer Konzentration von 1 bis 10 mM vorhanden ist.
6. Verfahren nach Anspruch 5, wobei das Phenylalaninamid in einer Konzentration von 10 mM vorhanden ist.
- 30 7. Verfahren nach Anspruch 3, wobei die menschlichen peripheren einkernigen Blutzellen oder die daraus entstandenen peripheren Blut-Lymphocyten in Gegenwart von Interleukin-2 kultiviert werden.
8. Verfahren nach Anspruch 5, wobei die menschlichen peripheren einkernigen Blutzellen oder die daraus entstandenen peripheren Blut-Lymphocyten in Gegenwart von Interleukin-2 kultiviert werden.
- 35 9. Verfahren nach Anspruch 7, wobei die menschlichen peripheren Blut-Lymphocyten, die durch das In-Berührung-Bringen mit dem Aminosäureamid erhalten werden, gewaschen und wieder suspendiert werden.
10. Verfahren nach Anspruch 8, wobei die menschlichen peripheren Blut-Lymphocyten, die durch das In-Berührung-Bringen mit Phenylalaninamid erhalten werden, gewaschen und wieder suspendiert werden.
11. Verfahren nach Anspruch 9, wobei die wieder suspendierten menschlichen peripheren Blut-Lymphocyten 2 bis 4 Tage lang in Gegenwart des rekombinanten Interleukin-2 kultiviert werden.
- 45 12. Verfahren nach Anspruch 10, wobei die wieder suspendierten menschlichen peripheren Blut-Lymphocyten 2 bis 4 Tage lang in Gegenwart des rekombinanten Interleukin-2 kultiviert werden.
13. Verfahren nach Anspruch 11, wobei die Konzentration der menschlichen peripheren Blut-Lymphocyten 50 3 x 10⁶ Zellen/ml bis 1 x 10⁸ Zellen/ml beträgt.
14. Verfahren nach Anspruch 12, wobei die Konzentration der menschlichen peripheren Blut-Lymphocyten 3 x 10⁶ Zellen/ml bis 1 x 10⁸ Zellen/ml beträgt.
- 55 15. Verfahren zur Herstellung von Lymphokin-aktivierten Killerzellen, in dem periphäre einkernige Blutzellen zur Herstellung einer Zellpopulation kultiviert werden, die für Tumorzellen, die gegen natürliche Killerzellen resistent sind, cytotoxisch sind, umfassend das In-Berührung-Bringen der peripheren einkernigen Blutzellen oder der daraus entstandenen peripheren Blut-Lymphocyten mit einer Kombina-

tion aus in m L-Aminosäureamid und einem L-Aminosäureni deralkyl ster, wobei das L-Aminosäur amid aus der aus Leucin, Isoleucin, Phenylalanin und Valin bestehenden Grupp ausgewählt ist und der L-Aminosäureniederalkylester aus der aus Alanin, Asparaginsäur , Cystein, Glutaminsäure, Glutamin, Phenylalanin, Prolin, Tyrosin, Tryptophan und Valin bestehenden Gruppe ausgewählt ist, und danach das Kultivieren der entstandenen Zellen.

- 5 16. Verfahren nach Anspruch 15, wobei die peripheren einkernigen Blutzellen menschliche Zellen sind.
- 10 17. Verfahren nach Anspruch 16, wobei das In-Berührung-Bringen für eine Zeitdauer von 20 bis 40 Minuten durchgeführt wird.
- 15 18. Verfahren nach Anspruch 17, wobei die L-Aminosäure Phenylalanin ist.
19. Verfahren nach Anspruch 18, wobei der Ester L-Phenylalaninmethylester und das Amid Phenylalanina- mid ist.
20. Verfahren nach Anspruch 19, wobei der Ester in einer Konzentration von 1 bis 5 mM vorhanden ist und das Amin in einer Konzentration von 1 bis 10 mM vorhanden ist.
- 25 21. Verfahren nach Anspruch 20, wobei das Salzsäure-Salz des Esters und des Amids vorhanden ist.
22. Verfahren nach Anspruch 19, wobei die menschlichen peripheren einkernigen Blutzellen oder periphären Blut-Lymphocyten in Gegenwart von Interleukin 2 kultiviert werden.
- 25 23. Verfahren nach Anspruch 20, wobei die menschlichen peripheren einkernigen Blutzellen oder periphären Blut-Lymphocyten in Gegenwart von Interleukin 2 kultiviert werden.
24. Verfahren nach Anspruch 22, wobei die peripheren Blut-Lymphocyten, die durch das In-Berührung-Bringen mit dem Ester und dem Amid erhalten werden, gewaschen und wieder suspendiert werden.
- 30 25. Verfahren nach Anspruch 23, wobei die peripheren Blut-Lymphocyten, die durch das In-Berührung-Bringen mit dem Ester und dem Amid erhalten werden, gewaschen und wieder suspendiert werden.
26. Verfahren nach Anspruch 24, wobei die wieder suspendierten peripheren Blut-Lymphocyten 2 bis 4 Tage lang in der Gegenwart des rekombinanten Interleukin 2 kultiviert werden.
- 35 27. Verfahren nach Anspruch 25, wobei die wieder suspendierten peripheren Blut-Lymphocyten 2 bis 4 Tage lang in der Gegenwart des rekombinanten Interleukin 2 kultiviert werden.
- 40 28. Verfahren nach Anspruch 26, wobei die Konzentration der peripheren Blut-Lymphocyten 3×10^6 Zellen/ml bis 1×10^8 Zellen/ml beträgt.
29. Verfahren nach Anspruch 27, wobei die Konzentration der peripheren Blut-Lymphocyten 3×10^6 Zellen/ml bis 1×10^8 Zellen/ml beträgt.

45 Revendications

1. Procédé de préparation de cellules tueuses activées par une lymphokine dans lequel les cellules mononucléaires du sang périphérique sont cultivées pour produire une population de cellules qui sont cytotoxiques pour les cellules tumorales résistantes aux cellules tueuses naturelles, caractérisé en ce qu'il comprend la mise en contact desdites cellules mononucléaires du sang périphérique ou des lymphocytes du sang périphérique en provenant, avec un amide d'acide aminé-L, l'acide aminé-L étant sélectionné dans l groupe consistant n leucine, isoleucine, phénylalanine, t valine, ou un mélange quelconqu d c s d rniers, puis la culture des c llules résultantes.
- 55 2. Un procédé selon la revendication 1, dans lequel lesdites cellules mononucléaires du sang périphérique sont des cellules humaines.

3. Un procédé selon la revendication 2, dans lequel l'étape de mise en contact est réalisée pendant une période de 40 minutes.
4. Un procédé selon la revendication 3, dans lequel l'acide aminé est la phénylalanine.
5. Un procédé selon la revendication 4, dans lequel l'amide de phénylalanine est présent à une concentration de 1 à 10 mM.
10. Un procédé selon la revendication 5, dans lequel l'amide de phénylalanine est présent à une concentration de 10 mM.
15. Un procédé selon la revendication 3, dans lequel les cellules mononucléaires du sang périphérique humain ou les lymphocytes du sang périphérique en provenant sont cultivés en présence d'interleukine-2.
20. 8. Un procédé selon la revendication 5, dans lequel les cellules mononucléaires du sang périphérique humain ou les lymphocytes du sang périphérique en provenant sont cultivés en présence d'interleukine-2.
25. 9. Un procédé selon la revendication 7, dans lequel les lymphocytes du sang périphérique humain obtenus par contact avec l'amidé d'acide aminé sont lavés et remis en suspension.
30. 10. Un procédé selon la revendication 8, dans lequel les lymphocytes du sang périphérique humain obtenus par contact avec un amide de phénylalanine sont lavés et remis en suspension.
35. 11. Un procédé selon la revendication 9, dans lequel les lymphocytes du sang périphérique humain remis en suspension sont cultivés pendant 2 à 4 jours en présence d'interleukine 2 recombinante.
40. 12. Un procédé selon la revendication 10, dans lequel les lymphocytes du sang périphérique humain remis en suspension sont cultivés pendant 2 à 4 jours en présence d'interleukine-2 recombinante.
45. 13. Un procédé selon la revendication 11, dans lequel la concentration en lymphocytes du sang périphérique humain est de 3×10^6 cellules/ml à 1×10^8 cellules/ml.
50. 14. Un procédé selon la revendication 12, dans lequel la concentration en lymphocytes du sang périphérique humain est de 3×10^6 cellules/ml à 1×10^8 cellules/ml.
55. 15. Un procédé de préparation de cellules tueuses activées par une lymphokine dans lequel les cellules mononucléaires du sang périphérique sont cultivées pour produire une population de cellules qui sont cytotoxiques pour les cellules tumorales résistantes aux cellules naturelles tueuses, caractérisé en ce qu'il comprend la mise en contact desdites cellules mononucléaires du sang périphérique ou des lymphocytes du sang périphérique en provenant avec une combinaison d'un amide d'acide aminé-L, et d'un ester d'alkyle inférieur d'acide aminé-L, l'amide de l'acide aminé-L étant sélectionné dans le groupe consistant en leucine, isoleucine, phénylalanine, valine, et l'ester d'alkyle inférieur d'acide aminé-L étant sélectionné dans le groupe consistant en alanine, acide aspartique, cystéine, acide glutamique, glutamine, phénylalanine, proline, tyrosine, tryptophane et valine, puis la culture des cellules résultantes.
60. 16. Un procédé selon la revendication 15, dans lequel les cellules mononucléaires du sang périphérique sont des cellules humaines.
65. 17. Un procédé selon la revendication 16, dans lequel l'étape de mise en contact est réalisée pendant une période de 20 à 40 minutes.
70. 18. Un procédé selon la revendication 17, dans lequel l'acide aminé-L est la phénylalanine.
75. 19. Un procédé selon la revendication 18, dans lequel l'ester est le méthyl ester de L-phénylalanine et l'amide est un amidé de la phénylalanine.

20. Un procédé selon la revendication 19, dans lequel l'ester est présent à une concentration de 1 à 5mM et l'amide est présent à un concentration des 1 à 10 mM.
- 5 21. Un procédé selon la revendication 20, dans lequel du chlorhydrate de l'ester et de l'amide sont présents.
22. Un procédé selon la revendication 19, dans lequel les cellules mononucléaires du sang périphérique humain ou les lymphocytes du sang périphérique sont cultivées en présence d'interleukine-2.
- 10 23. Un procédé selon la revendication 20, dans lequel les cellules mononucléaires du sang périphérique humain ou les lymphocytes du sang périphérique sont cultivées en présence d'interleukine-2.
24. Un procédé selon la revendication 22, dans lequel les lymphocytes du sang périphérique, obtenus par mise en contact avec l'ester et l'amide sont lavés et remis en suspension.
- 15 25. Un procédé selon la revendication 23, dans lequel les lymphocytes du sang périphérique, obtenus par mise en contact avec l'ester et l'amide sont lavés et remis en suspension.
26. Un procédé selon la revendication 24, dans lequel les lymphocytes du sang périphérique remis en suspension sont cultivés pendant 2 à 4 jours en présence d'interleukine-2 recombinante.
- 20 27. Un procédé selon la revendication 25, dans lequel les lymphocytes du sang périphérique remis en suspension sont cultivés pendant 2 à 4 jours en présence d'interleukine-2 recombinante.
28. Un procédé selon la revendication 26, dans lequel la concentration en lymphocytes du sang périphérique est de 3×10^6 cellules/ml à 1×10^8 cellules/ml.
29. Un procédé selon la revendication 27, dans lequel la concentration en lymphocytes du sang périphérique est de 3×10^6 cellules/ml à 1×10^8 cellules/ml.

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